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Original article

α-Aminoalkylphosphonates as a tool in experimental optimisation of P1 side chain shape of potential inhibitors in S1 pocket of leucine- and neutral aminopeptidases

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Abstract

The synthesis and biological activity studies of the series of structurally different α-aminoalkylphosphonates were performed in order to optimise the shape of the side chain of the potential inhibitors in S1 pocket of leucine aminopeptidase [E.C.3.4.11.1]. Analysis of a series of compounds with aromatic, aliphatic and alicyclic P1 side chains enabled to find out the structural features, optimal for that fragment of inhibitors of LAP. The most active among all investigated compounds were the phosphonic analogues of homo-tyrosine (K_i = 120 nM) and homo-phenylalanine (K_i = 140 nM), which even as racemic mixtures were better inhibitors in comparison with the best till now-phosphonic analogue of L-leucine (230 nM). Additional comparison of the inhibitory activity obtained for aminopeptidase N (APN, E.C.3.4.11.2) give insight into structural preferences of both enzymes.
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1. Introduction

Aminopeptidases are enzymes that catalyse the removal of amino acids from the N-terminus of a peptide or protein. These enzymes have been found in bacteria, yeast, plants, animal and human tissues, what is considerably ascribed to their broad substrate specificity [1]. They play such a biological functions as protein maturation, activation and degradation of bioactive peptides, hormone level regulation and control of cell proliferation [2,3]. It is well known, that elevated concentration of these enzymes was observed in such pathological disorders as cancer and cataracts [1,3]. In the case of the carcinogenesis, the methionine aminopeptidases has been shown to be a promising target for development of anti-angiogenesis agents [4]. Additionally, the importance of these enzymes arises from the observation, that some of them might play the role in the apoptosis of cancer cells, what makes them interesting targets in oncological research (with methionine and neutral aminopeptidases being the most promising targets) [5].

Aminopeptidases can be subdivided into two major groups, with the first one being responsible for the hydrolysis of amino acids with hydrophobic side chains from N-terminus of the peptide or protein, and the second, which specifically remove other amino acid residues [3].

Within the first group, leucine aminopeptidase (LAP, E.C.3.4.11.1) is one of the most detailed characterised enzyme with respect to its sequence, structure and mechanism of action. Human LAP plays an important role in the early stages of HIV infection, where the elevated concentration of this enzyme was observed [6]. The unusual activity of this enzyme has also been observed in such pathological disorders as cancer, inflammation of liver or eye cataract [7–9]. Because of this leucine aminopeptidase is often called the stress enzyme.

Substantial insight into the structure of the LAP derives from X-ray structures of the enzyme from bovine lens determined in its native form and complexed with transition state inhibitors such as: bestatin, amastatin, phosphonic analogue of L-leucine and L-leucinal coordinated to the active site.

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Bovine lens leucine aminopeptidase is a hexamer with a mass of 324 kDa, where every from six subunits is identical and contains three zinc ions. Two of them are located in active site of the enzyme and play a catalytic role, and third one is situated apart from the active site and most likely plays a structural function. LAP hydrolyses all of the substrates with N-terminal L-amino acid and glycine, but kinetic preference of the enzyme is towards L-leucine and L-phenylalanine, and to a less extend, to other hydrophobic substrates [10–15].

The inhibitory activity of the inhibitors of LAP is routinely measured using commercially available enzyme from porcine kidney. Unfortunately, X-ray structure of this enzyme has not been determined so far. In the search for the potential inhibitors of LAP this fact seems to have no significant meaning since both enzymes (from bovine lens and from porcine kidney) reveal high first-order sequence homology and similar kinetic properties, what suggests close structural features of their active centres [1].

Several sets of various inhibitors of leucine aminopeptidase have been reported so far. On the basis of their structure, they can be divided into two major groups. First one constitute analogues of short peptides, among which analogue of dipeptide-bestatine, and tetrapeptide-amastatine, are the best known ones [16,17]. The second group represent analogues of amino acids, with L-leucinal (K_i = 60 nM), the boronic acid analogue of L-leucine (K_i = 130 nM), phosphonic analogue of L-leucine (K_i = 230 nM) and hydroxamic analogue of D-leucine (K_i = 1.3 μM) being the most effective (Fig. 1) [18–22].

All of these inhibitors are transition state analogues of LAP, able to bind to two zinc ions present in the active site of the enzyme. According to the substrate specificity, the L-enantiomers at the N-terminus of polypeptide are stronger cooridinated to the active site of the enzyme than their D-enantiomers. Additionaly, the common feature of all presented inhibitors is the hydrophobic character of the side chain, where leucine isobutyl moiety is complexed by LAP.

The present results are the continuation of our previous studies on the inhibition of leucine aminopeptidase, where the crystal structure of L-leucine phosphonic acid coordinated in active centre of bovine lens leucine aminopeptidase was chosen as a lead compound in the search for the optimal structure of the inhibitors [23]. In the current study we have

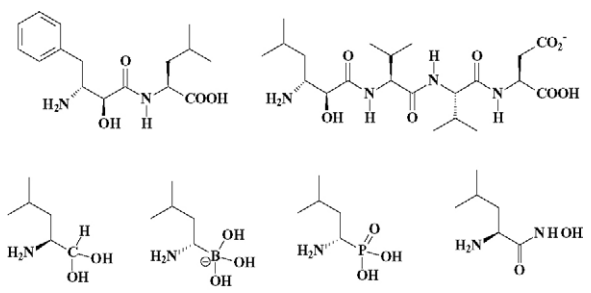

Fig. 1. The structures of the inhibitors of leucine aminopeptidase: bestatine, amastatine, L-leucinal, L-leucine boronic acid, L-L euP and D-leucine hydroxamic acid.

mainly focused on the experimental optimisation of the P1 side chain shape of the inhibitor located at S1 site of the enzyme, paying only marginal attention to the stereochemistry, because this feature has been the subject of earlier detailed investigations. Since similar comopunds have been reported as inhibitors of structurally closely related aminopeptidase N (APN, EC 3.4.11.2), we have performed additional studies to compare the structural requirements of S1 pocket of LAP versus the same pocket of APN [21].

2. Results

1-Aminoalkanephosphonic acids have been obtained as racemic mixtures according to the well established procedures. In the case of racemic compounds, the Oleksyszyn or modified Arbuzov methodologies have been applied. Enantiopure 1-aminoalkylphosphonates have been synthesised using Hamilton–Walker reaction (Table 1) [24–26]. In the case of 1-amino-3-phenylbutanephosphonic acid (**15**), the enzymatic assay was performed using the mixture of two pair of enantiomers obtained in 1:0.7 ratio (stereochemistry not determined) (Fig. 2).

Seven aromatic, three aliphatic and eight alicyclic aminoalkylphosphonates were used for optimisation of the P1 fragment of potential LAP inhibitors. These structures were selected according to the expected change of their inhibitory activity against LAP due to the carbon chain length, steric bulk, presence or absence of methylene bridge between aminophosphonate and hydrophobic portions of the molecule, as well as taking into consideration overall shape of the phosphonate. The introduction of the polar, hydroxy groups at the termini of long, hydrophobic chains was chosen to check the hypothesis that additional hydrogen bonds with Asp365 and Ala451 residues located at the bottom of S1 cavity might improve the binding properties of the potential inhibitor [23].

All of the 1-aminoalkylphosphonates tested in vitro using porcine kidney leucine aminopeptidase appeared to be slow-binding, competitive inhibitors of the enzyme with K_i values in the range of 0.12 and 798 μM. The mechanism of their binding is a slow one-step process, as indicated from the linear dependence of the apparent first-order rate constant for slow-binding versus inhibitor concentration The lowest activity was found for 1(RS)-amino-1-methyl-1-*iso*-propylmethanephosphonic acid (**2**) (K_i = 798 μM), an analogue od α-methylvaline. Both enantiomers of 1-amino-2-ethylbuthanephosphonic acid (**3/4**) (K_i = 243 and 119 μM for R and S enantiomer respectively) and 1(RS)-amino-3-propylpenthanephosphonic acid (**5**) (K_i = 33 μM) also rank among weak LAP inhibitors. Better results were obtained for alicyclic analogues since 1(RS)-amino-1-cyclopropylmethanephosphonic acid (**6**) (K_i = 84.5 μM), 1(RS)-amino-1-cyclobuthylmethanephosphonic acid (**7**) (K_i = 22.7 μM), 1(RS)-amino-4-cyclohexyl-buthanephosphonic acid (**20**) (K_i = 9.57 μM), both enantiomers of 1-amino-1-cyclohexylmethanephosphonic acid (**8/9**) (K_i = 7.89 and 6.96 μM for R

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